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Source: Journal of Wildlife Diseases, 45(4) : 1163-1168

Published By: Wildlife Disease Association

URL: <https://doi.org/10.7589/0090-3558-45.4.1163>

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Antibodies to West Nile Virus in Raccoons and Other Wild Peridomestic Mammals in Iowa

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ABSTRACT: Surveillance for evidence of West Nile virus (WNV) infection in small- and medium-sized wild mammals was conducted in Iowa, USA, from May 2005 to June 2007. Sera were collected from 325 mammals belonging to nine species and tested for antibodies to WNV and other flaviviruses by epitope-blocking enzyme-linked immunosorbent assay (ELISA). All sera that had antibodies to flaviviruses by blocking ELISA were further examined by plaque reduction neutralization test (PRNT). Thirteen mammals were seropositive for WNV by PRNT, including 10 raccoons (*Procyon lotor*). The seroprevalence for WNV in raccoons was 34%. Although a moderately high seroprevalence for WNV has been detected in raccoons in other surveillance studies in the United States, this has not been reported previously in Iowa or most bordering states. Together, these data indicate that raccoons are exposed to WNV at high rates throughout the United States. Two Virginia opossums (*Didelphis virginiana*) and one fox squirrel (*Sciurus niger*) were also seropositive for WNV. Nineteen mammals had antibodies to an undetermined flavivirus(es). In summary, we provide serologic evidence that raccoons in Iowa are commonly exposed to WNV.

Key words: Flavivirus, Iowa, mammals, *Procyon lotor*, raccoons, West Nile virus.

West Nile virus (WNV; family *Flaviviridae*; genus *Flavivirus*) is maintained in nature in an enzootic transmission cycle that primarily involves mosquitoes (*Culex* spp.) and birds (Hayes et al., 2005). Humans, horses, and other mammals are often regarded as incidental hosts because they usually develop viremias of insufficient magnitude to infect susceptible mosquitoes. Generally, an infected vertebrate must produce a viremia of $\geq 10^5$

plaque-forming units (pfu)/ml to serve as a reservoir host (Turell et al., 2000; Sardelis et al., 2001). Recent studies have demonstrated that some mammalian species, such as fox squirrels, eastern chipmunks (*Tamias striatus*), and golden hamsters (*Mesocricetus auratus*), develop WNV viremias sufficient to infect mosquitoes (Tesh et al., 2005; Tiawsirisup et al., 2005; Tonry et al., 2005; Root et al., 2006; Padgett et al., 2007; Platt et al., 2007; Gomez et al., 2008b). Although many WNV serosurveys have been performed on birds, few have been performed on wild mammals, particularly in the Western Hemisphere (Komar et al., 2001a, b; Gibbs et al., 2006). Thus, systematic serologic investigations are required to determine the host range and exposure rates of WNV in wild mammals. In a serosurvey performed in Iowa, USA, from 1999 to 2003, 2 yr before and after the first report of WNV activity in the state (CDC, 2009), antibodies to WNV were detected by plaque reduction neutralization test (PRNT) in 7.9–8.5% of white-tailed deer (*Odocoileus virginianus*) sampled in 2002–03 but in none sampled in 1999–2001 (Santaella et al., 2005). Because no other wildlife populations in Iowa have been serologically tested for evidence of WNV infection, we undertook a serosurvey to determine the host range and seroprevalence for WNV in small- to medium-sized wild mammals in Iowa.

Mammals were trapped in 11 study sites in western ($n=2$), central ($n=5$), and

eastern ($n=4$) Iowa from May 2005 through June 2007. Global positioning system locations of the study sites ranged from 41°57' to 42°47'N and from 91°54' to 96°38'W. Mammals were collected using Sherman folding and nonfolding aluminum live-traps (8.75×8.75×25 cm; H. B. Sherman Traps Inc., Tallahassee, Florida, USA) and three sizes of wire mesh collapsible Tomahawk live-traps (12.5×12.5×40, 15×15×47.5, and 30×30×100 cm; Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) to target a variety of small- to medium-sized mammals. Trapping was performed under a state of Iowa permit. Each site was trapped on seven occasions; three times each in 2005 and 2006 and once in 2007. Traps were baited with peanut butter, apples, rolled oats, or canned cat food to target a variety of small- to medium-sized mammals. Deer mice (*Peromyscus maniculatus*), eastern chipmunks, and fox squirrels were anesthetized using a cotton pledget saturated with isoflurane (Abbott Laboratories, Abbott Park, Illinois, USA). All other mammals were immobilized with ketamine: acepromazine, which was given intramuscularly at 44:10 mg/kg. Whole blood was collected from the lateral saphenous or jugular vein of all live-trapped animals with the exception of mice and chipmunks, which were bled from the retro-orbital plexus. Blood was collected from the heart of carcasses of an American beaver (*Castor canadensis*) and muskrat (*Ondatra zibethicus*) provided by a local fur trapper. Small mammals (i.e., deer mice) were marked by toe clip, and large mammals (i.e., raccoons and opossums) were marked by ear tag to identify recaptures then released alive. Samples were transferred to BD Microtainer tubes (BD Biosciences, San Jose, California, USA) and placed on wet ice (0 C). Samples were then transported to Iowa State University (ISU), centrifuged, and stored at -70 C. All animals were handled and treated in accordance with experimental protocols approved by the ISU Animal Care and Use Committee.

Sera were screened at 1:10 for antibodies to flaviviruses by epitope-blocking enzyme-linked immunosorbent assay (ELISA) as described previously (Blitvich et al., 2003a, b). ELISAs were performed using the WNV-specific monoclonal antibody (MAb) 3.1112G (Millipore Bioscience Research Reagents, Temecula, California, USA), or the flavivirus group-reactive MAb 6B6C-1 (InBios International, Seattle, Washington, USA). Both MABs are produced in house mice (*Mus musculus*). MAb 6B6C-1, which is labeled with horseradish peroxidase (HRP), was used to test all sera. In contrast, MAb 3.1112G is not labeled with HRP; therefore, an HRP-labeled anti-mouse (*Mus*) reporter antibody was used in the WNV-specific ELISA. Consequently, the WNV-specific assay was not used to test sera from house mice, although sera from all other species were tested. Antigen was prepared from WNV-infected *Aedes albopictus* (C6/36) cell cultures. The ability of the test sera to block the binding of the MABs to WNV antigen was compared with the blocking ability of control serum without antibody to flaviviruses. Data are expressed as relative percentages, and inhibition values $\geq 30\%$ are considered as indicating the presence of viral antibodies.

All sera positive for antibodies to flaviviruses by blocking ELISA were tested by PRNT to identify the infecting virus. PRNTs were conducted according to standard methods in the Biosafety Level 3 facilities at ISU (Beaty et al., 1995). PRNTs were done using WNV (strain NY99-35261-11) and St. Louis encephalitis virus (SLEV; strain TBH-28) with African green monkey kidney (Vero) cells. SLEV was included in these experiments because it has been identified in Iowa and is known to react with antibodies to WNV (Calisher et al., 1989; Gubler et al., 2007). Viruses were obtained from the World Health Organization Center for Arbovirus Reference and Research maintained at the Centers for Disease Control and Prevention, Division of Vector-Borne Infectious

Diseases, Fort Collins, Colorado, USA. Sera were initially tested at a dilution of 1:20, and titers are expressed as the reciprocal of serum dilutions yielding $\geq 90\%$ reduction in the number of plaques (PRNT₉₀). For etiologic diagnosis, the PRNT₉₀ antibody titer to the respective virus was required to be at least fourfold greater than that to the other flavivirus tested.

Sera were collected from 325 mammals belonging to nine species. The most common species were deer mouse ($n=215$), eastern chipmunk ($n=32$), house mouse ($n=32$), raccoon ($n=29$), and Virginia opossum ($n=13$). One of each of the following also was sampled: an American beaver, fox squirrel, groundhog (*Marmota monax*), and muskrat. The majority ($n=226$) of mammals were sampled in 2005; others were sampled in 2006 and 2007 ($n=89$ and 10, respectively). Thirty-two (9.8%) mammals had evidence of flavivirus infection by blocking ELISA (Table 1). All 32 mammals seemed healthy when captured. The flavivirus-positive mammals consisted of raccoons ($n=14$), deer mice ($n=9$), Virginia opossums ($n=5$), eastern chipmunks ($n=3$), and a fox squirrel ($n=1$). With exception of those that only had a single animal tested, the species with highest seroprevalence for flaviviruses were raccoon (48%) and Virginia opossum (38%). The flavivirus antibody prevalences for mammals sampled in 2005, 2006, and 2007 were 11.9%, 5.6%, and 0%, respectively, although only a small number ($n=10$) of mammals was sampled in 2007.

Thirteen mammals with blocking ELISA antibodies to flaviviruses were considered seropositive for WNV because their WNV PRNT₉₀ titers were at least fourfold greater than their corresponding titers to SLEV (Table 1). None of the mammals were seropositive for SLEV. The mammals seropositive for WNV by PRNT consisted of raccoons ($n=10$), Virginia opossums ($n=2$), and a fox squirrel ($n=1$), and the WNV seropreva-

lence rates for these species were 34%, 15%, and 100%, respectively, although only a single fox squirrel was sampled. There was little change in the seroprevalence for WNV in mammals in 2005 and 2006 (4.4% and 3.3%, respectively). Furthermore, there was no change in the annual number of WNV cases in humans in Iowa during this time; 37 cases occurred in both 2005 and 2006 (CDC, 2009). None of the mammals sampled in 2007 had antibodies to WNV but, as noted above, this is presumably because the sample size was small.

Raccoons, Virginia opossums, and fox squirrels also were demonstrated to have WNV-specific antibody in a serosurvey conducted in 2003 in the central and eastern United States (Root et al., 2005). More recently, antibodies to WNV were identified in 29% of fox squirrels sampled in 2005–06 in Colorado (Root et al., 2007). In Maryland, USA, 67% of raccoons and 52% of Virginia opossums sampled in 2005–06 had antibodies to WNV (Gomez et al., 2008a). A high (46%) seroprevalence for WNV also was reported for raccoons in a serosurvey conducted in 2003–04 in the western and central United States (Bentler et al., 2007). In Iowa, the seroprevalence for WNV in white-tailed deer in 2002–03 was 11.2–12.7% by blocking ELISA and 7.9–8.5% by PRNT (Santaella et al., 2005).

Nineteen (5.8%) mammals had antibodies to an undetermined flavivirus(es) (Table 1). Antibodies to an undetermined flavivirus(es) also were identified in white-tailed deer in Iowa (Santaella et al., 2005). The seroprevalence for the undetermined flavivirus was considerably higher in deer sampled in 2002–03 (10.6–20.7%) than in 1999–2001 (2.2–3.2%). These findings could indicate that a subset of animals considered to have antibodies to an undetermined flavivirus(es) had been exposed to WNV but did not meet the PRNT criterion for a WNV infection. Indeed, two mammals sampled in this study (IA-109 and IA-229) had moderately high WNV PRNT₉₀

TABLE 1. Mammals with epitope-blocking ELISA or neutralizing antibodies to flaviviruses.^a

Sample ID	Sampling date	Common name	Blocking ELISA ^b		PRNT ₉₀ titer		Final diagnosis
			3.1112G ^c	6B6C-1 ^d	WNV	SLEV	
IA-19	5/05	Chipmunk	— ^e	36	—	—	FLAVI ^f
IA-244	7/06	Chipmunk	—	30	—	—	FLAVI
IA-250	7/06	Chipmunk	50	—	—	20	FLAVI
IA-18	5/05	Deer mouse	—	32	—	—	FLAVI
IA-20	5/05	Deer mouse	—	45	—	20	FLAVI
IA-21	5/05	Deer mouse	—	35	—	—	FLAVI
IA-111	10/05	Deer mouse	—	92	—	20	FLAVI
IA-123	10/05	Deer mouse	—	56	—	—	FLAVI
IA-150	9/05	Deer mouse	—	32	—	—	FLAVI
IA-164	8/05	Deer mouse	—	36	—	—	FLAVI
IA-166	8/05	Deer mouse	—	53	—	20	FLAVI
IA-175	10/05	Deer mouse	—	31	—	—	FLAVI
IA-240	7/06	Fox squirrel	61	83	≥640	80	WNV ^g
IA-22	7/05	Opossum	—	38	—	—	FLAVI
IA-105	8/05	Opossum	—	31	160	80	FLAVI
IA-162	9/05	Opossum	—	57	80	40	FLAVI
IA-239	7/06	Opossum	44	76	≥640	80	WNV
IA-297	8/06	Opossum	35	42	≥640	160	WNV
IA-2	5/05	Raccoon	38	63	80	20	WNV
IA-3	5/05	Raccoon	49	38	80	20	WNV
IA-9	6/05	Raccoon	—	60	80	40	FLAVI
IA-15	5/05	Raccoon	35	82	≥640	80	WNV
IA-16	5/05	Raccoon	34	61	320	40	WNV
IA-23	7/05	Raccoon	—	35	20	—	FLAVI
IA-124	8/05	Raccoon	—	93	—	20	FLAVI
IA-126	8/05	Raccoon	—	76	320	80	WNV
IA-131	8/05	Raccoon	—	54	160	40	WNV
IA-132	8/05	Raccoon	—	93	160	40	WNV
IA-194	9/05	Raccoon	—	72	160	40	WNV
IA-223	9/05	Raccoon	37	38	160	40	WNV
IA-224	9/05	Raccoon	39	79	≥640	80	WNV
IA-229	11/05	Raccoon	—	83	160	80	FLAVI

^a ELISA = enzyme-linked immunosorbent assay; PRNT = plaque reduction neutralization test; WNV = West Nile virus; SLEV = St. Louis encephalitis virus; MAb = monoclonal antibody.

^b Inhibition values ≥30% are considered significant.

^c MAb 3.1112G is WNV-specific.

^d MAb 6B6C-1 is flavivirus-specific.

^e Virus-specific antibodies not detected.

^f An animal was considered to have antibodies to an undetermined flavivirus if the serum sample was positive in at least one serologic assay but a definitive diagnosis was not made by PRNT.

^g An animal was considered to be seropositive for WNV if its WNV PRNT₉₀ titer was at least fourfold greater than the corresponding titer to SLEV. The PRNT is the standard in arbovirus serology; thus, the PRNT data were used to determine whether an animal was seropositive for WNV.

titers (160 in each case), but because these titers were only twofold greater than the corresponding SLEV PRNT₉₀ titer, they were considered to have antibodies to an undetermined flavivirus. Another possibility is that a flavivirus, other than WNV or

SLEV, is currently active in Iowa. The flavivirus could be a member of the Modoc antigenic complex because several viruses in this complex (i.e., Cowbone Ridge, Sal Vieja, San Perlita, and Modoc viruses) were first isolated in mammals (Kuno et al.,

1998). Another possibility is that the mammals had been exposed to an unrecognized flavivirus. To explore these possibilities, more than 6,000 mosquitoes (*Culex* and *Aedes* spp.) collected in 2007 in multiple sites in Iowa were assayed by reverse transcription-polymerase chain reaction (RT-PCR) using flavivirus-specific primers. Of the 340 mosquito pools tested, 51 were positive for *Culex* flavivirus (Blitvich, unpubl. data), a recently discovered flavivirus that seems to be insect-specific (Hoshino et al., 2007). However, none of the mosquitoes had been infected with a flavivirus that could account for the serologic findings reported here. Another possibility is that the mammals had been exposed to a flavivirus transmitted by an atypical vector (e.g., ticks; de la Fuente et al., 2008). To address this issue, ticks removed from vertebrate hosts in Iowa are currently being screened in our laboratory by RT-PCR using flavivirus-specific primers.

The majority (66%) of mammals sampled in this study were deer mice. Nine (4.2%) deer mice had flavivirus-specific antibody, but none had been exposed to WNV (Table 1). A much higher (41%) flavivirus seroprevalence was reported in deer mice in the central and eastern United States in 2003, and none had antibodies to WNV (Root et al., 2005). The difference in the prevalence of flavivirus antibody in these studies could be due to regional variations in vector feeding preferences. Another explanation is that deer mice sampled by Root et al. (2005) had been infected with a flavivirus not present in Iowa.

In summary, antibodies to WNV were identified in several mammalian species, including raccoons, sampled in 2005–07 in Iowa. The seroprevalence for WNV in raccoons was 34%. Moderate to high WNV seroprevalences also have been reported for raccoons in other serosurveys in the United States (Root et al., 2005; Bentler et al., 2007; Gomez et al., 2008a). Because of the peridomestic behavior of raccoons, and recent reports that other

mammalian species develop WNV viremia $\geq 10^{5.0}$ pfu/ml, experimental infection studies should be performed to investigate the potential of raccoons to serve as reservoir hosts of WNV. More specifically, research is needed to determine the duration and magnitude of viremia, and clinical signs and mortality rates (if any) in raccoons inoculated with US strains of WNV.

This study was supported by grant R01 C1000267-01 from the US Centers for Disease Control and Prevention and by start-up funds provided by Iowa State University.

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Received for publication 20 January 2009.